Effects of Diclofop and Diclofop-Methyl on the Membrane Potentials of Wheat and Oat Coleoptiles

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ABSTRACT

Electrophysiological measurements were made on the mesophyll cells of wheat (Triticum aestivum L. cv Waldron) and oat (Avena sativa L. cv Garry) coleoptiles treated either with the herbicide diclofop-methyl (methyl 2-(4-(2',4'-dichlorophenoxy)phenoxy)propanoate), or its primary metabolite diclofop, (2-(4-(2',4'-dichlorophenoxy)phenoxy)propanoic acid). Application of a 100 micromolar solution of diclofop-methyl to wheat coleoptiles had little or no effect on the membrane potential (E_m), however in oat, E_m slowly depolarized to the diffusion potential (E_o). At pH 5.7, 100 micromolar diclofop rapidly abolished the electrogenic component of the membrane potential in both oat and wheat coleoptiles with half-times of 5 to 10 minutes and 15 to 20 minutes, respectively. The concentrations giving half-maximal depolarizations in wheat were 20 to 30 micromolar compared to 10 to 20 micromolar in oat. The depolarizing response was not due to a general increase in membrane permeability as judged from the E_m's response to changes in K^+, Na^+, Cl^-, and SO_4^{2-}, before and after treatment with diclofop and from its response to KCN treatment. In both plants, diclofop increased the membrane permeability to protons, making the E_m strongly dependent upon the external pH in the range of pH 5.5 to pH 8.5. The effects of diclofop can best be explained by its action as a specific proton ionophore that shuttles protons across the plasmalemma. The rapidity of the cell's response to both diclofop-methyl (15–20 minutes) and diclofop (2–5 minutes) makes the ionophoric activity a likely candidate for the earliest herbicidal event exhibited by these compounds.

Diclofop-methyl (methyl 2-(4-(2',4'-dichlorophenoxy)phenoxy)propanoate) is a selective postemergence grass herbicide that controls wild oat and other grasses in wheat (1). The reasons for its phytotoxicity and selectivity are not known for certain. The selectivity is probably due to a differential metabolism between susceptible and tolerant species (8). In both susceptible oats and resistant wheat, diclofop-methyl is rapidly hydrolyzed to the free acid, diclofop (2-(4-(2',4'-dichlorophenoxy)phenoxy)propanoic acid. In wheat diclofop is subsequently metabolized by aryl hydroxylation and conjugation to form an acidic, aryl-glucoside; in oat, diclofop is metabolized to a neutral glucosyl ester (7, 14, 21, 23). The reason for the tolerance of wheat to the herbicide is attributed to the metabolism and modification of the ring structure of diclofop as compared to ester formation in oat. It is not known for certain whether the toxic form of the herbicide is diclofop-methyl, diclofop, or both.

The reason for the phytotoxicity and the mechanism of action of diclofop-methyl is less well understood than is the metabolism. In susceptible plants such as oat, diclofop-methyl induced an inhibition of leaf, stem, and root elongation, chlorosis of leaves (11), and severe damage to cellular ultrastructure (2). After 92 h treated leaves contained chloroplasts with compressed grana thylakoids and indistinct envelopes while the remainder of the cytoplasm had extensive vesiculation similar to that seen in senescent cells (2). Simultaneous application of diclofop-methyl and auxin herbicides such as 2,4-D under field conditions resulted in a reduction in herbicidal activity of both diclofop-methyl and the auxic compound (6). Shimabukuro et al. (22) have reported an antagonism of auxin-induced growth responses by diclofop-methyl in whole plants and in tissue culture. In oat but not wheat coleoptiles, IAA induced elongation and proton extrusion were inhibited 1 h after treatment with diclofop-methyl (21). Crowley and Frendeville (4) reported that discs from wild oat, barley, and wheat leaves treated for 12 h with diclofop-methyl had a higher rate of leakage of electrolytes than untreated leaves, suggesting that some form of membrane damage had occurred. Cohen and Morrison (3) using mitochondria isolated from both wheat and oat found that diclofop was a more potent inhibitor of state 3 respiration than was diclofop-methyl, appeared to uncouple state 4 respiration, and enhanced the rate of passive swelling of mitochondria in isotonic KCl medium, indicating a direct influence of diclofop on the permeability properties of the inner mitochondrial membranes.

The results from various studies suggest that the herbicidal effects of diclofop-methyl or its primary metabolite diclofop, involved an increase in plasmalemma and mitochondrial membrane permeability, antagonism of auxin mediated processes and extensive disruption of the cytoplasm. However, all of these studies used relatively long treatment times, and almost certainly involved secondary effects, or they used isolated organelles. To determine the initial, primary effects, which may be the common link to all the results discussed above, measurements of cell membrane electrical potentials were used as indicators of rapid changes in membrane activities.

MATERIALS AND METHODS

To study the mode of action of diclofop-methyl and diclofop, peeled coleoptiles of oat (Avena sativa L. cv Garry), a susceptible plant, and wheat (Triticum aestivum L. cv Waldron), a resistant plant were used. Seeds of each were surface sterilized for 5 min in 1% sodium hypochlorite, rinsed for 1 h in running tap water, then were placed in paper rag dolls held upright in tap water and germinated in an incubator as described previously (20). Because the oat and wheat seeds germinated at different rates, uniform coleoptiles 25 to 30 mm in length were obtained by germinating oat seeds for 3 d and wheat seeds for 4 d at 26°C. Seeds were exposed to 4 h of red light/d to inhibit the elongation of the oat mesocotyls.

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The coleoptiles used for these experiments had the apical 3 mm removed, were debladed and peeled using fine forceps, after which they were incubated in Higinbotham's 1X nutrient solution (10) for a minimum of 2 h prior to the start of electrical measurements. In those experiments where the pH was varied, the phosphate buffer was replaced by a 1 mM Mes-Tris buffer; all other ion concentrations were as in the 1X nutrients. The 2 h pretreatment time was necessary to allow the membrane potentials to stabilize after the coleoptiles were peeled.

For an experiment a coleoptile was held in a plexiglass tissue chamber and constantly irrigated with nutrient solution. Membrane potential measurements were made using glass microelectrodes inserted into individual cells (5). The electrical measurements were monitored with a Mentor N-9502 intracellular probe system connected to an oscilloscope and strip chart recorder. The glass electrodes used had a resistance of 15 to 20 megaohms and tip potentials of less than 15 mV. The tip potentials were nullled using the Mentor probe system. To begin an experiment a cell was impaled with an electrode and the potential allowed to stabilize before applying the treatment solutions.

Because of the limited solubility of diclofop-methyl, stable suspensions were prepared by pipetting an appropriate amount of a stock solution (usually 20 mM in acetone) into a volumetric flask and enough reagent grade acetone added to give a final acetone concentration of 1% (v/v). With constant agitation, buffer solutions were slowly added to the flask to the desired volume. The resultant translucent herbicidal suspensions obtained in this way were stable for several hours at room temperature.

Because of the greater water solubility of diclofop (the acid), treatment solutions were prepared by adding the appropriate volume of a stock solution in acetone directly into the buffer. Both control and treatment solutions contained 1% (v/v) of acetone. Composition of other solutions were as given in the figure legends. Unless stated otherwise all solutions were at pH 5.7.

The $E_D$ values were estimated by application of 2 mM KCN to the bathing medium until the cells were maximally depolarized.

The figures presented are typical results chosen to show relative rates and magnitudes. Each experiment was repeated at least three times.

Samples of diclofop-methyl and diclofop were obtained as described previously (15).

RESULTS

Impalement of oat or wheat coleoptile cells by microelectrodes gave rapid measurements of the membrane potentials. If the impalement was made shortly after the coleoptiles were peeled the potentials were found to become more negative over a period of 30 to 60 min, after which they remained constant for several hours. For this reason a pretreatment time of 2 h was chosen to allow the potential to stabilize. The average membrane potential of oat cells was $-116$ mV $\pm 18$ mV ($n = 141$) and of wheat cells was $-112$ mV $\pm 13$ mV ($n = 38$). Application of KCN to the bathing medium caused rapid depolarizations of the measured $E_M$. The $E_D$ was estimated as the value at maximum depolarization. The average estimated $E_D$ for oat was $-34$ mV $\pm 8$ mV ($n = 10$) and for wheat was $-54$ mV $\pm 12$ mV ($n = 8$). Both $E_M$ and $E_D$ were in agreement with previously published values for oat coleoptiles (10, 19).

Coleoptiles of wheat and oat differed in their response to diclofop-methyl but were very similar in their response to diclofop. Application of a 100 $\mu$M solution of diclofop-methyl to wheat coleoptiles had very little effect on the $E_M$ (Fig. 1). An initial transient perturbation of the potential which stabilized at or near the pretreatment value of $-100$ mV was observed. In contrast, treatment of oat coleoptiles resulted in a slow decline in the $E_M$ to $-35$ mV, the estimated $E_D$ (10, 19), after an initial lag of 10 to 15 min.

A $100\mu$M solution of diclofop, the free acid, at pH 5.7 induced rapid depolarizations in both wheat and oat. In oat the $E_M$ depolarized very rapidly between $-100$ mV and $-45$ mV, followed by a transient hyperpolarization of approximately $10$ mV. After the transient hyperpolarization and a short period of depolarization, measurements were often very difficult to obtain either continuously in the same cell or by insertion of the electrode into adjacent cells. Usually at this time, movement of organelles was visible in the cells, followed by large increases in the electrode resistance. The large increases in resistance were probably caused by occlusion of the electrodes since small pieces of material could often be seen clinging to the tips after withdrawal from the cells. In those cases where the depolarization could not be measured continuously, measurements taken 15 to 20 min after the onset of treatment gave values at or near $E_D$. Occasionally, cells could be measured continuously and were found to have a transient hyperpolarization followed by a slower depolarization to $E_D$ as shown in Figure 1.

In wheat coleoptiles the diclofop-induced depolarizations were less rapid and lacked the transient hyperpolarizations seen in oat. Continuous measurements from $E_M$ to $E_D$ were easily made without the perturbations observed previously. In wheat the depolarization was complete in 40 to 45 min.

Because diclofop is the primary metabolite of diclofop-methyl and because it induced rapid depolarizations in both oat and wheat, its effect was studied in greater detail. Figure 2 shows the concentration dependence of the diclofop-induced depolarizations. Both wheat and oats were maximally depolarized by 100 $\mu$M diclofop. However, wheat appeared to be much less sensitive than oat. The concentration required to cause a half-maximal depolarization in wheat was 20 to 30 $\mu$M compared to 10 to 20 $\mu$M in oats. Also, the maximal depolarization was only three-fourths that of oat.

To determine whether or not the diclofop induced depolarizations were due to a structural disruption of the membrane and a concomitant increase in permeability as suggested by Prendiville et al. (4, 18) or to some other unexamined mode of action, the effects of varying the external ion concentration were measured before and after treatment with diclofop. Figure 3 shows the relationship between the external K$^+$ concentration and the depolarization of oat cells when K$^+$ is increased above 1 mM. In untreated tissue increasing K$^+$ (as K$_2$SO$_4$) from 10 to 100 mM induced a depolarization of 20 to 25 mV. After application of 100 $\mu$M diclofop the $E_M$ depolarized to $-35$ mV, the estimated $E_D$ but had little or no effect on the K$^+$ induced depolarizations. The slightly lower depolarizations after diclofop treatment may be explained by a decreased movement of K$^+$ across the membrane in response to the lower charge density inside the cell. The same results were obtained for KCl, K$_2$SO$_4$, and NaCl (data not shown).

Application of 2 mM KCN to oat coleoptiles induced a rapid depolarization of $E_M$ from $-125$ mV to $-30$ mV (Fig. 4). Diclofop applied at this time induced no further change in the measured potential.

When oat and wheat coleoptiles were incubated in Mes-Tris buffer for 2 h at pH 8.5, the $E_M$ was polarized to $-160$ mV in wheat and $-130$ mV in oat (Fig. 5). Subsequent decreases in pH
FIG. 1. Recorder tracings illustrating the response of the membrane potentials of oat and wheat coleoptiles to treatment with 100 μM diclofop-methyl (DM) or 100 μM diclofop at pH 5.7. The coleoptiles had the epidermis removed and were allowed to equilibrate in 1× solution for 2 h prior to the start of electrical measurements. Treatment solutions were applied at the arrow.

FIG. 2. Concentration dependence of the diclofop-induced depolarizations in oat (○) and wheat (□). The data points represent the average of at least three separate experiments (ΔEm = final Em minus initial Em).

FIG. 3. Effects of 100 μM diclofop on the potassium-induced depolarization of oat cells. The peeled coleoptiles were preincubated in buffer containing 0.5 mM K2SO4 for 2 h. With control cells the K+ was varied as K2SO4 and the level of depolarization recorded. In the case of treated cells, electrical measurements were initiated, followed by application of 100 μM diclofop. After the cells were maximally depolarized, external potassium was varied as for control cells. Each point represents the average of three experiments (ΔEm = final Em minus initial Em).
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FIG. 4. Effects of diclofop on the diffusion potential. Oat coleoptiles were treated with 2 mM KCN as indicated. After the cells had maximally depolarized the buffer was changed to one containing both 2 mM KCN and 100 µM diclofop at pH 5.7.

FIG. 5. Effects of the pH of the bathing solution before and after treatment with 100 µM diclofop. Coleoptiles were incubated at pH 8.5 for 2 h prior to the start of electrical measurements. Diclofop was added initially at pH 8.5. Subsequent changes in pH below 8.5 in the presence of diclofop induced depolarizations of the Em to new equilibrium values. The points represent the values at equilibrium. Wheat control (□), wheat treated (■), oat control (○), oat treated (○).

from 8.5 to 5.5 had little or no effect on the Em of control coleoptiles. Diclofop at pH 8.5 had little or no effect on Em but when the pH was decreased below 8.5 the Em changed rapidly to a new equilibrium value. At approximately pH 6 for wheat and 5.5 for oat, Em was maximally depolarized to Ed. The potential measured at any given pH was often constant for up to an hour and was readily reversible, i.e. changing from pH 5.5 to pH 8.5 induced a hyperpolarization. The depolarization rate observed with a decrease in pH was faster (e.g. 3 mV min⁻¹) than the hyperpolarization rate (e.g. 1 mV min⁻¹) observed with an increase in pH. The value of the Em in the presence of diclofop was dependent upon the pH regardless of the initial Em (data not shown).

Discussion

In the present work diclofop-methyl had little or no effect on the membrane potentials of wheat, a resistant plant, yet induced a slow depolarization of the potential in oat cells. This difference in activity probably reflects not only the rate of hydrolysis of the ester to form the more toxic free acid in oat but also the rapid detoxication of diclofop by aryl hydroxylation and subsequent glycosylation in wheat. Shimabukuro et al. (21) have reported that in peeled wheat coleoptiles, after 20 min, 51% of the diclofop-methyl applied remained as the ester while the remainder had been metabolized to diclofop (40%) and other metabolites (9%); after 2.5 h 9% was present as the parent ester, 27% as diclofop, and the remainder to further metabolites. In comparison, oat coleoptiles appeared to hydrolyze the ester more rapidly (75% of the label applied was diclofop and 23% as diclofop-methyl after 20 min), yet after 2.5 h 90% was still present as the acid and 3% as the ester. The resistance of wheat to the herbicide can be seen within minutes following treatment with the parent ester. The resulting free acid-induced depolarizations observed in both plants indicate that the rate of hydrolysis of diclofop-methyl and the subsequent metabolism of the acid are important factors in the resistance of wheat to the herbicide.

The effects of the primary metabolite, diclofop, on the electrical properties of the membranes of both wheat and oat coleoptiles can best be explained by diclofop acting primarily and quite specifically as a proton ionophore (15) rather than to increase the general permeability properties of these membranes as shown not to be the case in Figures 3 and 4. The response of the membrane potential to changes in external ion concentrations is dependent upon the concentration gradient of the ions across the membrane and the permeability of the membrane (9, 25). Diclofop did not alter the response of the Em to K⁺, Cl⁻, SO₄²⁻, or Na⁺, the ions present at the highest concentrations in the cells (10, 19). An argument could be made that these ions were near enough to their diffusion equilibria that alterations in membrane permeability would not be detected readily.

To circumvent this argument we reasoned that since the Em can be thought of as having two components, the component due to passive diffusion of the major ions (Ed), and a metabolically dependent, electric component (Ep) (25), inhibition of
respiration by KCN and the abolishment of $E_d$ would make $E_m$ more indicative of, if not equal to, $E_d$. Changes in $E_m$ then would be due to changes in membrane permeability. Application of KCN followed by diclofop (Fig. 4) had no effect on $E_m$. If diclofop had nonspecifically altered the membrane permeability, the $E_m$ would have depolarized to near zero under these conditions.

Since diclofop did not appear to affect passive membrane transport properties, it must have affected the $E_d$ of the potential. In higher plants $E_d$ is thought to be a proton translocating ATPase that establishes and maintains a pH gradient between the cytoplasm and cell free space (24, 25). In control cells at pH 8.5, changes in the external pH had little or no effect on $E_m$ (Fig. 5) suggesting that the membranes were relatively impermeable to protons. Application of diclofop at pH 8.5 had little effect on either wheat or oat coleoptiles. However, when the pH was lowered in the presence of diclofop, $E_m$ responded to changes in pH. The $E_m$ was dependent on the pH of the treatment solution until the cells were maximally depolarized to $E_d$. Similar pH effects were seen with DNP (data not shown). The depolarizations at pH values lower than 8.5 were not attributed to diclofop acting as a weak acid (15).

The responses of wheat and oat cells to diclofop were very similar to those observed in Chara cells (15). In Chara at pH 5.7, diclofop induced a rapid depolarization of $E_m$ followed by a transient hyperpolarization and subsequent further depolarization much like that seen with oat. Measurements of membrane resistance indicated that immediately following treatment with diclofop, membrane resistance decreased when the depolarization rate was the fastest followed by an increase in resistance to 65% of the pretreatment value as the $E_d$ reached a minimum. The effects of diclofop were attributed to its action as a proton ionophore and not as a weak acid. In Chara, 1 mM propionic acid induced a transient hyperpolarization of 10 to 12 mV as would be expected for a weak acid. Application of 100 μM diclofop induced a rapid depolarization of the membrane potential in Chara (15), wheat, and oat. At pH 5.7 approximately 0.75% of the diclofop applied would be present in the neutral, protonated form. The concentrations used were therefore probably too low to cause such a transient hyperpolarization as would be expected from diclofop acting simply as a weak acid.

The physicochemical similarities of diclofop, DNP, and CCCP as well as their effects on membrane associated functions suggest that diclofop may also function as a proton ionophore. Diclofop, DNP, and CCCP are lipophilic weak acids with similar pKa values (3.57, 3.9, and 5.65, respectively) and lipid solubilities (log octanol-water partition coefficients of 3.5, 1.5, and 3.2, respectively). By comparison, propionic acid has a pKa of 4.9 and a partitioning coefficient of less than 0.5. Diclofop, CCCP, and DNP induce depolarizations of the membrane potential and reductions in cytoplasmic ATP content with similar time courses.

In addition, diclofop did not appear to inhibit the proton ATPase in vivo since it did not inhibit the spontaneous repolarization of $E_m$ following application of 50 mM sucrose; in vitro diclofop increased the K+ stimulated, pH 6, ATPase activity of isolated oat root microsomes by 30 to 40% (unpublished data).

The observed herbicidal and physiological effects and the selectivity of diclofop-methyl between wheat and oat may be explained by the proposed model of diclofop activity as shown in Figure 6. In both plants diclofop-methyl is rapidly hydrolyzed to the free acid by esterases present in the cell free space or in the cytoplasm. The acid may be conjugated to an inactive bound form (glucose ester) or detoxified by argyl glucoside formation (14, 23). The lipid solubility of both the ester and the acid allow them to partition into the membrane phase where the acid-base dissociation characteristics (pKa = 3.57) (15), the pH gradient, and the charge difference across the membrane causes diclofop to function as a proton ionophore. By analogy to FCCP and DNP (17), diclofop in its neutral, protonated form (DH) diffused across the membrane and dissociated in response to the pH gradient, and released the proton into the cytoplasm. The negatively charged diclofop (D−), repelled by the negative membrane potential, diffused back across the membrane where it again accepted a proton. As long as a proton electrochemical gradient existed, D− tended to cycle catalytically to collapse the potential and to decrease the ATP levels as the proton translocating ATPase attempted to maintain the potential (17, 26, 27).

The net result would be to short circuit and stimulate the ATPase (15), following the depolarization of the membrane potential as we have observed. This could lead to a reduction in cellular ATP levels and an alteration in cytoplasmic pH. Lucas et al. (15) reported that after treatment of Chara cells with diclofop for 90 min, the ATP concentration of the cytoplasm was reduced to only 3.6% of the control at pH 5.7, 63.4% at pH 7.0, and no reduction occurred at pH 8.2. Although values for the effects of diclofop on cytoplasmic pH are lacking, an effect can be deduced from the work of Shimabukuro et al. (21). They reported that peeled oat and wheat coleoptiles tended to acidify the medium from pH 6.2 to pH 5.6 or lower. Diclofop induced a net alkalinization of the medium from 6.2 to pH 6.8, indicating a net movement of protons from the external solution into the cells. Further consequences would be as has been observed, i.e. to antagonize the effects of compounds that promote proton extrusion from the cells such as auxins (5, 18, 21, 22, 28) and fusicoxan (unpublished data), to inhibit cell elongation (16, 20, 21), to disrupt or alter metabolism (12, 13), to disrupt cellular ultrastructure (2), and the loss of membrane integrity (4, 18).

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